Exhibit A

Journal of Applied Microbiology 2002, **93**, 302–309

Antibacterial activity of surfactants against *Escherichia coli* cells is influenced by carbon source and anaerobiosis

S. Ishikawa, Y. Matsumura, K. Katoh-Kubo and T. Tsuchido

Department of Biotechnology, Faculty of Engineering, Kansai University, Yamate-cho, Suita, Japan

2001/376: received 30 November 2001, revised 15 April 2002 and accepted 24 April 2002

S. ISHIKAWA, Y. MATSUMURA, K. KATOH-KUBO AND T. TSUCHIDO. 2002.

Aims: In order to clarify the involvement of an energy-yielding system in the antibacterial action of surfactants, the effects of carbon source and anaerobiosis during the growth period on the surfactant sensitivity of *Escherichia coli* cells were investigated.

Methods and Results: Cetyltrimethylammonium bromide (CTAB) and *N*-dodecyl-*N*,*N*-dimethylglycine, at relatively low concentrations, caused a delay in growth of *E. coli* cells. Cells grown in M9 medium supplemented with glycerol, succinate or acetate as a carbon source were more sensitive to surfactants and had a higher respiratory activity than those grown with glucose. Cultivation under anaerobiosis made cells resistant to CTAB.

Conclusions: Bacterial sensitivity to surfactants was affected by carbon source and anaerobiosis.

Significance and Impact of the Study: The results obtained should be helpful in determining suitable conditions of treatment in the practical use of surfactants for bacterial decontamination.

INTRODUCTION

Since surfactants are amphophilic compounds which decrease the surface tension of solutions (Porter 1994), they have been used in various industries, hospitals and the home as wetting agents, emulsifiers, solubilizing agents, foaming and antifoaming agents, detergents, etc. Among them, cationic surfactants such as quaternary ammonium compounds and, although less active, zwitterionic surfactants possess antimicrobial activity at relatively low concentrations (Merianos 1991; Hugo and Russell 1992).

Surfactants interact with various cellular components, in particular proteins and lipids, and consequently cause deleterious effects on the growth and viability of microbial cells (Merianos 1991; Hugo and Russell 1992). Surfactants also induce cell autolysis in *Bacillus subtilis* (Tsuchido *et al.* 1985, Tsuchido *et al.* 1987; Tsuchido *et al.* 1990) and inhibit respiration by *Pseudomonas aeruginosa* cells (Majtan *et al.* 1995). It remains unclear, however, which cellular component is the critical target for cell survival and growth.

Several surfactant-resistant and -sensitive strains of bacteria have been isolated by artificial or spontaneous mutagenesis and reported to possess altered structures and functions in their cell envelope (Nishikawa *et al.* 1979; Vaara 1981; Sakagami *et al.* 1989; Heir *et al.* 1995, 1998; Lacroix *et al.* 1996). Vaara (1981) has reported that novel lipid A mutants of *Salmonella typhimurium* are more sensitive than their parent strains to benzalkonium chloride. Sakagami *et al.* (1989) have suggested that the increases in quantities of cellular membrane components, such as phospholipids, fatty acids and neutral lipids, suppress the adsorption of benzalkonium chloride molecules to the cell. The present authors have also isolated and characterized a cationic surfactant-resistant mutant of *Escherichia coli* and found that the strain has an altered cell envelope compared with its parent strain (Ishikawa *et al.* 2002). In addition to the envelope change, an altered ability of efflux pumps for surfactants has also been proposed as a mechanism of cellular acquired resistance to surfactants (Heir *et al.* 1995, 1998; Lacroix *et al.* 1996).

Nickerson and colleagues (Kramer *et al.* 1980; Kramer and Nickerson 1984 and Kramer *et al.* 1984) have reported that a sodium dodecyl sulphate (SDS)-resistant strain isolated from *Enterobacter cloacae* requires much larger amounts of energy source and oxygen for growth in SDS-containing medium and suggested that SDS attacks the cell membrane, especially membrane proteins. Subsequently, they have pointed out that SDS may also cause osmotic

Correspondence to: Dr T. Tsuchido, Department of Biotechnology, Faculty of Engineering, Kansai University, Yamate-cho 3-3-35, Suita 564-8680, Japan (e-mail: ttsuchi@ipcku.kansaiu.ac.jp).

stress to cells, since they used high concentrations of SDS to inhibit cell growth (Adamowicz *et al.* 1991; Nickerson and Aspendon 1992).

The antimicrobial activity of surfactants is influenced not only by the chemical properties of surfactants but also by how a target micro-organism has grown before and during the surfactant treatment. Among factors affecting the latter situations, the effects of carbon source and anaerobiosis on the surfactant action seems not to have been much characterized (Russell 1992). These factors may change the energy metabolism and also modify cell structure and, therefore, should profoundly affect the bacterial sensitivity to surfactants.

In this study, in order to obtain some understanding of the mechanism of the antibacterial action of surfactant and also to obtain information for the practical use of surfactants for bacterial decontamination, the sensitivities of *E. coli* cells to a cationic surfactant, cetyltrimethylammonium bromide (CTAB), and some other surfactants were investigated using different carbon sources and in the absence and presence of oxygen.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Escherichia coli OW6 Pro^-, a derivative of strain W3110, was mainly used (Kitagawa *et al.* 2000). A *cya* mutant of *E. coli*, strain CA8306 (Kumar 1976), and its parent strain CA8000 were also employed in part of the experiments. Cells were grown overnight at 37°C in M9 salts supplemented with 0·2% glucose, unless otherwise stated, as a carbon source and, if necessary, 0·5 mmol l^{-1} L-proline. In the experiment on carbon source effect, glucose, glycerol, sodium succinate or sodium acetate was added at a final concentration of 1% to M9 salts. To examine the effect of cyclic adenosine 3′,5′-monophosphate (cAMP), it was added at a final concentration of 1 mmol l^{-1} to the medium.

A volume (2 ml) of the above culture was inoculated into a 500-ml flask containing 100 ml medium, the composition of which was the same as for precultivation, to give an $O.D._{650}$ of 0·03 and an aqueous solution of surfactant was then added to the medium. The flask was shaken at 37 °C at 120 rev min^{-1}. In some experiments, cells were cultivated in a Bioscanner, an automatic growth-recording incubator (OT-BS-48; Ohtake Works, Tokyo, Japan), by using L-shaped glass tubes (Tsuchido and Takano 1988). When cultivated anaerobically, a rubber-sealed cylindrical glass vessel (6·5 cm diam. and 26·5 cm length) was employed with bubbling at 1 v.v.m. of a gas mixture consisting of 90% nitrogen, 5% carbon dioxide and 5% hydrogen. In this experiment, precultivation was also performed similarly.

Measurement of respiratory activity

Respiratory activity was determined from the rate of consumption of dissolved oxygen monitored by a Clark-type oxygen electrode (YSI model 5300; Yellow Springs Instrument, Yellow Spring, OH, USA). The *in vivo* respiration assay was performed on cells cultivated as described above. The *in vitro* assay was performed as follows. Log-phase cells were collected by centrifugation, washed with 10 mmol l^{-1} *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid buffer (HEPES buffer, pH 7·0) and then resuspended in fresh buffer to give an $O.D._{650}$ of the suspension of 3·0. Cells were disrupted twice through a French Press (10 000 psi; Thermo Spectronic, New York, NY, USA) and the resultant cell homogenate then centrifuged at 4300 *g* to remove unbroken cells. Glucose (5 mmol l^{-1}) or NADH (3 mmol l^{-1}) was used as a substrate for respiration for the *in vivo* or *in vitro* assay, respectively.

Cyclic adenosine 3′,5′-monophosphate assay

Cultivated cells were treated with 7·5% trichloroacetic acid and then sonicated immediately (Sonifier Model 250; Branson Ultrasonics, Danbury, CT, USA). After cell debris was removed by centrifugation (1500 *g*, 10 min), cAMP in this supernatant fluid was extracted four times with water-saturated diethylether and the solvent remaining in the sample then evaporated by heat treatment at 70°C for 5 min. The cAMP content of this sample was measured by using a cyclic AMP immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA). The protein content was measured by the Bradford method (Bradford 1976).

Chemicals

Surfactants, including CTAB, and other chemicals used in this study were purchased from Wako Pure Chemical Industries (Osaka, Japan), except for *N*-dodecyl-*N*,*N*-dimethylglycine (DDMG) which was a gift from Dr H. Kourai (University of Tokushima, Japan).

RESULTS

Cell growth in the presence of surfactants

The aerobic growth of *E. coli* in M9 medium containing glucose as a carbon source was inhibited by the surfactants tested. When a cationic surfactant (CTAB) at 5 μmol l^{-1} or a zwitterionic type (DDMG) at 100 μmol l^{-1} was added at the start of cultivation, the initiation of cell growth was delayed without affecting the cell yield (Fig. 1). Substantial cell lysis was seen with cells treated with CTAB or

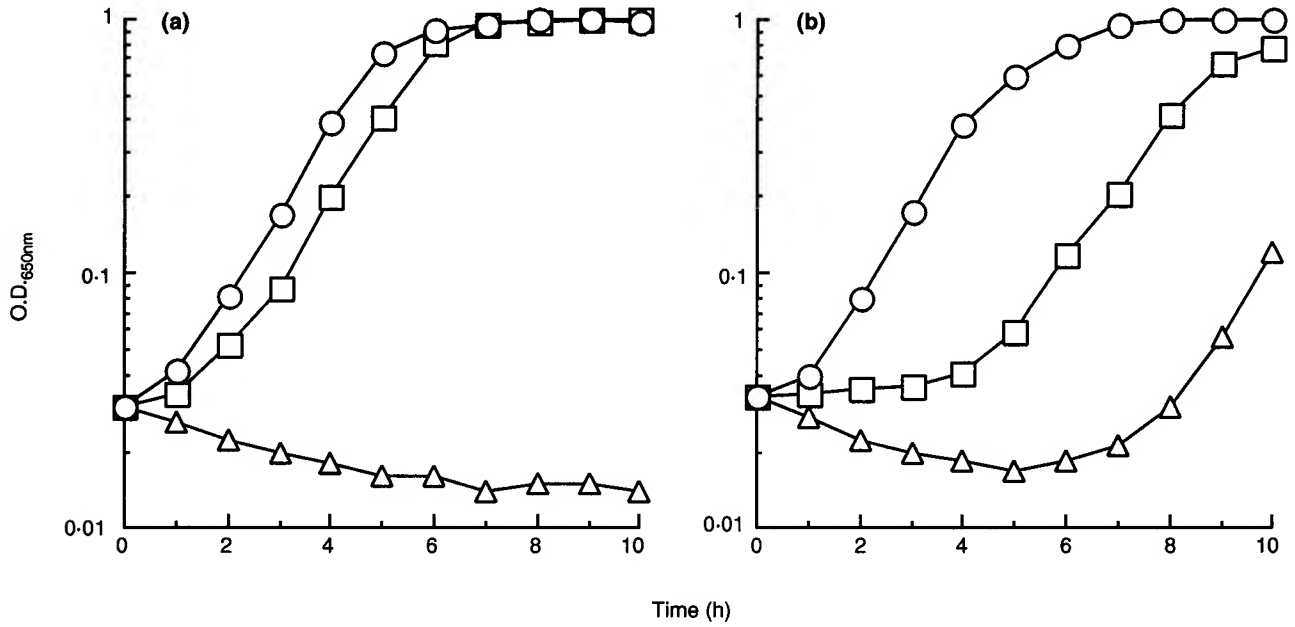

Fig. 1 Effect of surfactants on the growth of *Escherichia coli* OW6 in the presence of 0·2% glucose. (a) Cetyltrimethylammonium bromide at concentrations of 5 (□) and 10 (△) μmol l^{-1}. (b) *N*-dodecyl-*N*,*N*-dimethylglycine at concentrations of 100 (□) and 120 (△) μmol l^{-1}. ○, Control

DDMG at a concentration above 10 or 120 μmol l^{-1}, respectively, as detected by both the reduction in $O.D._{650}$ and microscopy (data not shown). Lauric acid and glycerol monolaurate, as anionic and non-ionic types, respectively, had no effect on the growth rate at 100 μmol l^{-1}, although they lowered the cell yield to some extent and the latter caused cell lysis after the treated culture entered the stationary phase of growth (data not shown). In further experiments, therefore, CTAB and DDMG were employed.

Effect of carbon source on the surfactant sensitivity

Nickerson and co-workers (Kramer *et al.* 1984; Aspedon and Nickerson 1993a, 1993b) have reported that the sensitivity of *Ent. cloacae* to SDS is affected by the type of carbon source for cell growth and also that SDS gives rise to an increase in respiratory activity and thus in an energy burden. Therefore, the effect of carbon source on their surfactant sensitivity was also examined using *E. coli* cells.

The CTAB sensitivity was compared among cells grown in M9 medium containing glucose, glycerol, succinate and acetate at 1·0%. As a result, glycerol-grown cells were found to be more sensitive to CTAB than glucose-grown cells (Fig. 2b). Likewise, succinate- and acetate-grown cells were also more sensitive to CTAB than glucose-grown cells (Fig. 2c and d). Similar results were also obtained with DDMG (data not shown). These results indicate that the surfactant sensitivity of *E. coli* cells is affected by the type of carbon source in the growth medium.

Effect of cyclic adenosine 3′,5′-monophosphate on the surfactant sensitivity

The type of carbon source for cell growth is known to affect the intracellular level of cAMP in *E. coli* (Botsford 1981; Botsford and Harman 1992). Therefore, the effect of cAMP addition on the CTAB sensitivity of *E. coli* OW6 cells was examined. When cAMP was added, at a final concentration of 1 mmol l^{-1}, to the growth medium containing 0·2% glucose for both precultivation and subsequent cultivation, cell growth was markedly inhibited by 5 μmol l^{-1} CTAB and 80 μmol l^{-1} DDMG, while cells grown without cAMP were only slightly affected (Fig. 3). Such a sensitization effect of added cAMP was also observed with strain CA8000 (data not shown). No substantial influence of cAMP itself on cell growth was observed.

To further investigate the relationship of cAMP to surfactant sensitivity, a *cya* mutant, CA8306, which possessed no substantial amount of cAMP, was used (Kumar 1976). The intracellular cAMP content was measured and estimated to be 0·4 pmol (μg protein)$^{-1}$ for strain CA8306 and 2·2 pmol (μg protein)$^{-1}$ for strain CA8000. Although this low intracellular cAMP content of the *cya* mutant was expected to make cells resistant to CTAB, compared with its parental strain, no significant effect of the *cya* mutation could be observed (Fig. 4).

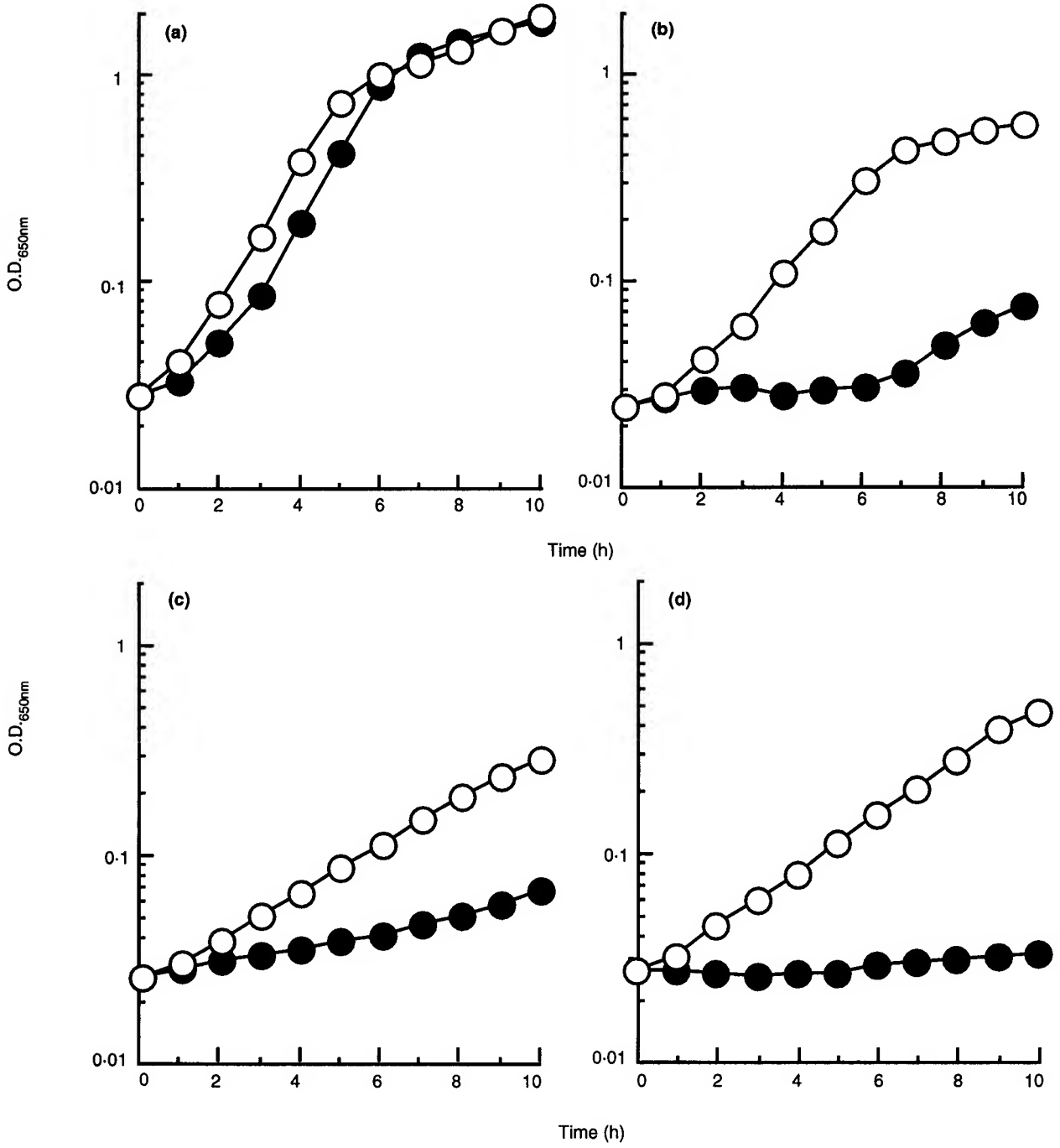

Fig. 2 The cetyltrimethylammonium bromide (CTAB) sensitivity of OW6 cells grown in the presence of (a) glucose, (b) glycerol, (c) succinate and (d) acetate at a concentration of 1·0%, respectively. CTAB at 5 μmol l^{-1} (●) was added at time zero. ○, Control

Cetyltrimethylammonium bromide sensitivity of anaerobically-grown cells

Since a more drastic change in the cellular energy-yielding system should be caused by cultivation under aerobic or anaerobic conditions (Patschkowski *et al.* 2000), the surfactant sensitivity was compared between cells grown under these conditions (Fig. 5). Although cells grown aerobically were lysed by CTAB at 10 μmol l^{-1}, under anaerobic conditions cells were able to grow in the presence of this concentration of CTAB.

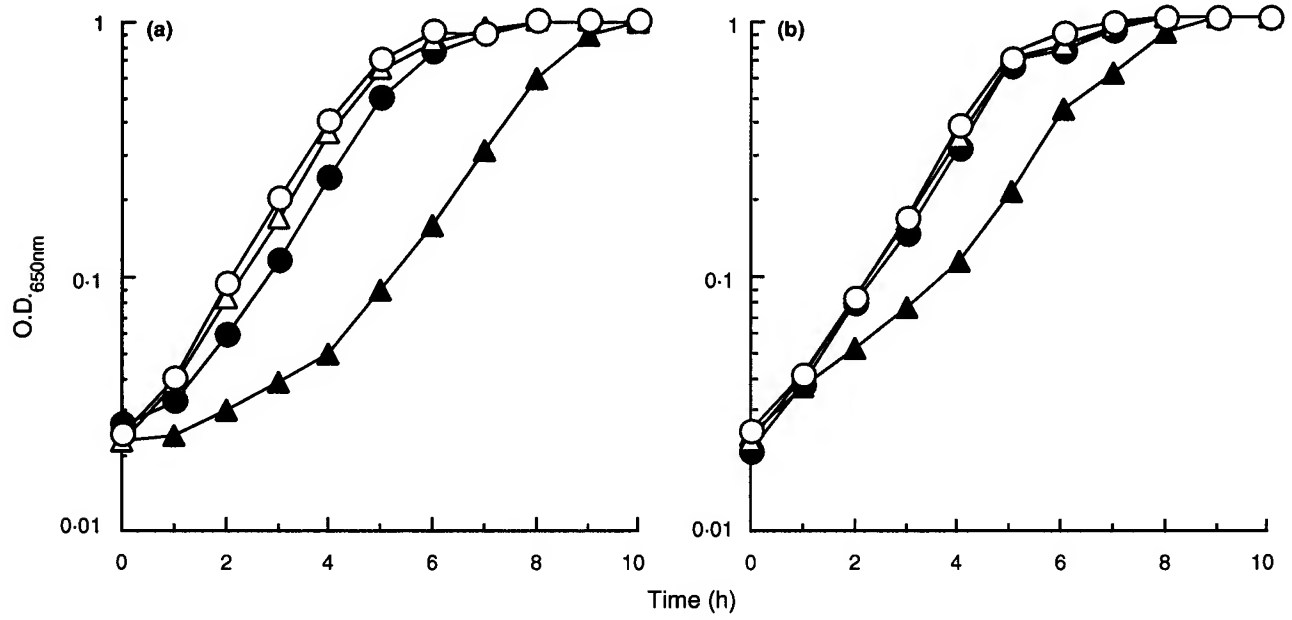

Fig. 3 Effect of exogenous cyclic adenosine 3′,5′-monophosphate (cAMP) on surfactant sensitivity of *Escherichia coli* OW6. Cells were grown in the presence of 0·2% glucose with (△, ▲) or without (○, ●) 1 mmol l^{-1} cAMP and also with (closed symbols) or without (open symbols) the addition of cetyltrimethylammonium bromide (CTAB) or *N*-dodecyl-*N*,*N*-dimethylglycine (DDMG). (a) CTAB or (b) DDMG were added at a concentration of 5 or 80 μmol l^{-1}, respectively

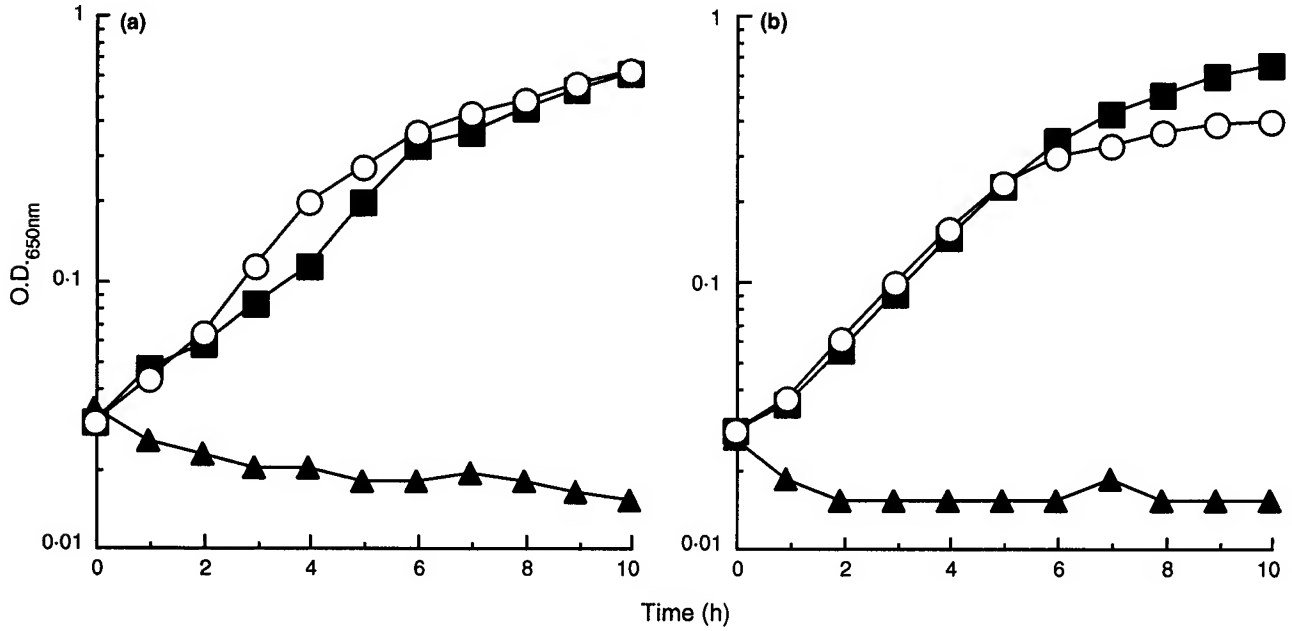

Fig. 4 The cetyltrimethylammonium bromide (CTAB) sensitivity of *Escherichia coli* (a) CA8000 and (b) CA8306. Cells were grown in the presence of 0·2% glucose with or without the addition of CTAB. CTAB was added to cultures at the following concentrations: 0 (○), 5 (■) and 10 (▲) μmol l^{-1}

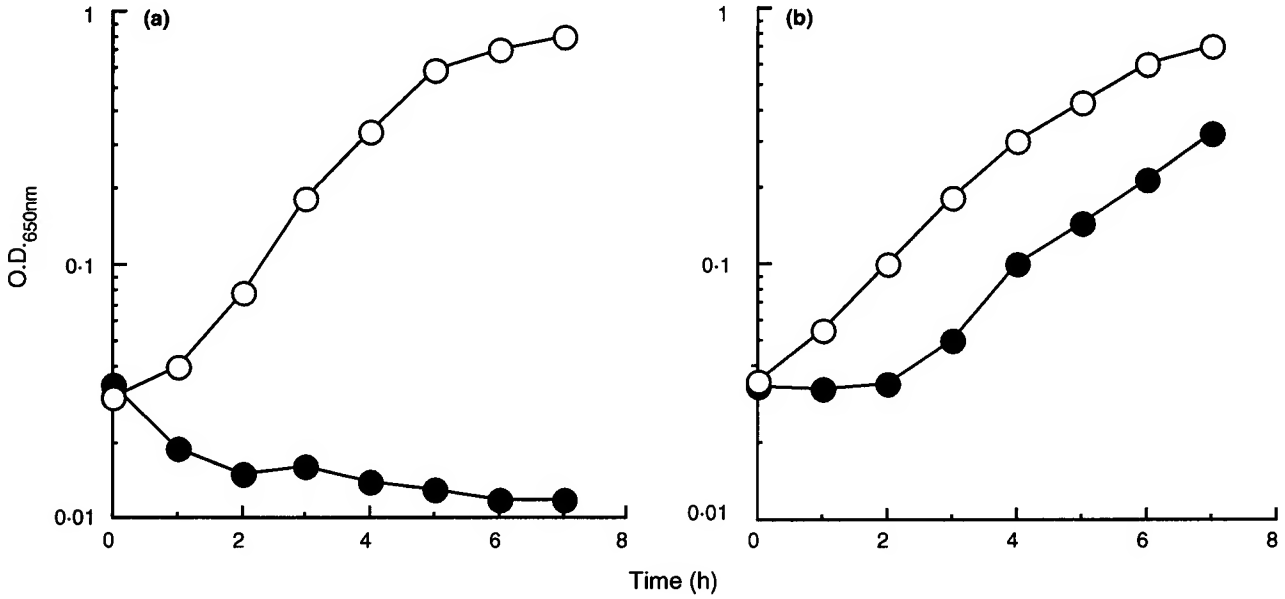

Fig. 5 The cetyltrimethylammonium bromide (CTAB) sensitivity of *Escherichia coli* OW6 under (a) aerobiosis and (b) anaerobiosis. Cells were grown in the presence of 0·2% glucose with (●) or without (○) 10 μmol l^{-1} CTAB

Inhibitory effect of cetyltrimethylammonium bromide on respiratory activity

To measure the respiratory activity, cells were grown to log phase in M9 salts with 0·2% glucose and then treated with CTAB (Fig. 6a). The activity was immediately decreased to approx. 80% by the addition of CTAB at 10 μmol l^{-1}. At a higher concentration of CTAB, 40 μmol l^{-1}, the activity was reduced to approx. 30%. When the respiratory activity of the cell homogenate was examined using NADH as a substrate, the activity was decreased to approx. 60% after the addition of CTAB even at 10 μmol l^{-1} and diminished at 30 μmol l^{-1} (Fig. 6b).

DISCUSSION

Although bacterial resistance to surfactants is profoundly influenced by cellular physiology (Hugo and Russell 1992), the effect of the carbon source on the surfactant sensitivity seems to have scarcely been investigated. Several workers have reported that the carbon source modulates the components of the cell membrane and cellular functions including respiration and, as a result, alters the bacterial sensitivities to some disinfectants and polymyxin B (Conrad *et al.* 1979; Russell 1992). It seems likely that the marked difference observed here in the sensitivities of *E. coli* to CTAB and DDMG between glucose and glycerol as carbon sources for cell growth is due to alteration of the cell envelope structure. This may lead to reduced permeation of surfactant molecules across the membranes and also lower the level of surfactant molecules localizing at the membranes.

It is well known that glucose as a carbon source reduces the intracellular level of cAMP in *E. coli* (Botsford 1981; Botsford and Harman 1992). In the present study, a possible relationship was found between the cellular cAMP content and the surfactant sensitivity of *E. coli*, as follows. Both cultivation in glycerol and the addition of cAMP to the culture sensitized cells to surfactants although, in the *cya* mutant, a relatively low intracellular cAMP content did not make cells resistant to CTAB. Since cAMP has a pleiotropic effect on cell physiology, including membrane functions, its effect on the surfactant sensitivity of cells should be rather complicated.

No published reports have been found concerning the effect of anaerobiosis on bacterial sensitivity to surfactants. The low sensitivity of cells grown anaerobically, compared with cells grown aerobically, may be explained by cellular inability to perform oxygen respiration. For cells grown aerobically, the suppression of function of the electron transport system localizing at the cytoplasmic membrane, essential for efficient energy yielding, should be critical for cell growth. The surfactants may disturb the membrane structure through interaction with phospholipids as well as membrane proteins (Merianos 1991; Hugo and Russell 1992), including respiratory enzymes, and, as a consequence, lower the respiratory activity, as confirmed here.

Some investigators have already reported that, in *E. coli* cells, the cellular respiratory activity is increased by the replacement of glucose with glycerol as a carbon source

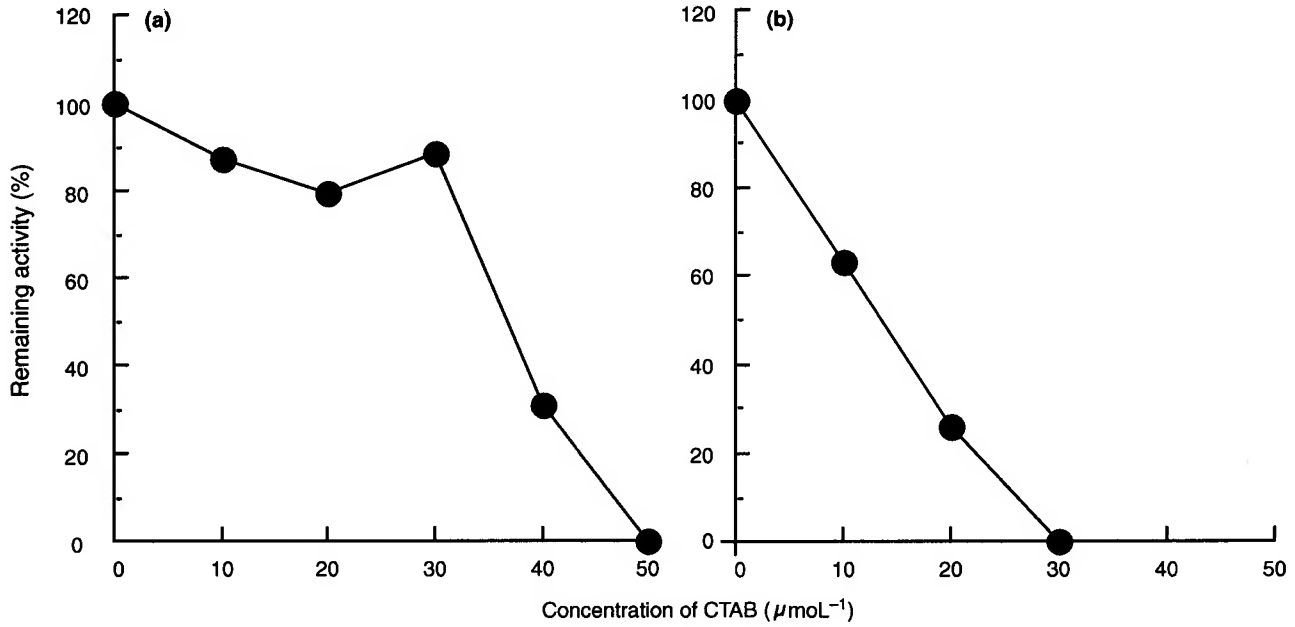

Fig. 6 The effect of cetyltrimethylammonium bromide (CTAB) on the respiratory activities of (a) whole cells and (b) the cell homogenate of *Escherichia coli* OW6. The respiratory activity was measured immediately after the addition of CTAB. The initial activities were taken as 100%, being 19·2 (nmol O_2) min^{-1} (mg dry wt cell homogenate)$^{-1}$ for the cell homogenate and 100 (nmol O_2) min^{-1} (mg dry wt cell)$^{-1}$ for whole cells. Values are means of results from two independent experiments

(Hempfling and Mainzer 1975; Takahashi 1975). The present authors have previously observed a high respiratory activity with glycerol-, succinate- and acetate-grown cells compared with glucose-grown cells (unpublished data) and, in such cells, the surfactant sensitivity was correspondingly high, as indicated in the present study.

It is also known that a global network of genes functions in *E. coli* cells grown anaerobically, i.e. the FNR and Arc regulatory systems, including *fumB* (fumarase B), *cyd* (cytochrome *d* oxidase) and *narGHJI* (nitrate reductase) (Patschkowski *et al.* 2000). It cannot be ruled out, therefore, that anaerobiosis may alter some cellular function or structure other than the cellular oxidation and respiration systems, as also described for the effect of carbon source described above, that makes cells less sensitive to surfactants.

It is unclear whether the variations in the surfactant sensitivity observed in this study result from independent influences of carbon source and anaerobiosis or some interrelated mechanism between these factors. However, the authors prefer the idea that some common machinery is involved in the cellular sensitivity as influenced by the above factors. Based on the above correlation between the cellular sensitivity to surfactants and respiratory activity as affected by carbon source and also the result of the respiration inhibition by CTAB, it is speculated that one of the possible targets of the surfactant action may be the respiratory system and the energy-transducing system linked to it in aerobically growing cells of *E. coli*. Surfactants may interfere with the activity (or activities) of some respiratory enzyme(s) and/or the energy coupling machinery which is (are) critical for cell growth in the presence of oxygen. Further studies are necessary to understand the detailed mechanisms of the surfactant action.

ACKNOWLEDGEMENTS

The authors thank Dr H. Kourai (University of Tokushima, Japan) for the gift of DDMG.

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